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The *Drosophila* RGS protein Loco is required for dorsal/ventral axis formation of the egg and embryo, and nurse cell dumping

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Abstract

The *loco* gene encodes members of a family of RGS proteins responsible for the negative regulation of G-protein signalling. At least two transcripts of *loco* are expressed in oogenesis, *loco-c2* is observed in the anterior-dorsal follicle cells and is downstream of the epidermal growth factor receptor signalling pathway, initiated in the oocyte. *loco-c3* is a new transcript of *loco*, which is expressed in the nurse cells from stage 6 onwards. Analysis of newly generated mutants and antisense technology enabled us to establish that disrupting *loco* in follicle cells results in ventralized eggs, while disrupting *loco* in nurse cells results in short eggs, due to defective dumping of the nurse cell cytoplasm into the oocyte.

Keywords: Oogenesis; Follicle cells; Epidermal Growth Factor Receptor signalling; G-protein signalling; Polarity

1. Introduction

A fertilised egg has the ability to give rise to a complete organism, with all its different cell types arranged into tissues, organs and systems. To achieve this, the oocyte has to be highly organised, and may contain localised maternal products that, in some organisms, direct early events and cell divisions in the embryo. Without these molecules positioned correctly, an embryo is unable to develop normally. The highly organised patterning of the *Drosophila* oocyte is established during oogenesis and is dependent on cooperation between the oocyte, other germ-line cells and associated somatic cells.

The mechanisms underlying the determination of anterior–posterior (AP) and dorsal–ventral (DV) polarity have been intensively researched in recent years (e.g. Nusslein-Volhard, 1991; Roth and Schüpbach, 1994; Gonzales-Reyes et al., 1995; Roth et al., 1995). Despite our increased knowledge there are still gaps in our understanding. For example, many components interacting with the *gurken-torpedo* signalling pathway including *rhomboid*, *kekkon*, *argos*, *pointed* and *Broad-complex* have been described (Ruohola-Baker et al., 1993; Neuman-Silberberg and Schüpbach, 1994; Roth and Schüpbach, 1994; Deng and Bownes, 1997; Wasserman and Freeman, 1998; Ghiglione et al., 1999; Zhao and Bownes, 1999), but we still have not established how all these components fit together and cooperate to establish DV polarity and dorsal-anterior egg structures.

In egg chambers at stage 8 of oogenesis *gurken* (*grk*) mRNA localises at the posterior of the oocyte, where it is translated. Grk protein signals to the adjacent follicle cells, which adopt a posterior fate as opposed to a default, anterior fate (Gonzalez-Reyes et al., 1995). These posterior follicle cells signal back to the oocyte resulting in the repolarisation of microtubules within the oocyte (Gonzalez-Reyes et al., 1995). It is the correct polarisation of the oocyte cytoskeleton that is responsible for establishing the AP axis of the embryo (Pokrywka, 1995). The maternal mRNAs *bicoid* and *nanos* are positioned at the poles of the egg and are responsible for initiating AP patterning within the embryo (Nusslein-Volhard et al., 1987; Berleth et al., 1988; Nusslein-Volhard, 1991). The AP axis of the egg is identified by anterior structures such as the micropyle and operculum, while the posterior is more rounded and the chorion is smooth.

After repolarisation of the microtubules the nucleus migrates anteriorly during stages seven to eight (Gonzalez-Reyes and St Johnston, 1994). g*rk* mRNA localises at the nucleus and protein produced locally signals to adjacent follicle cells again. Torpedo (TOP, the Epidermal Growth

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Factor Receptor (EGFR) homologue (Schüpbach, 1987; Clifford and Schüpbach, 1989; Roth et al., 1995; Sapir et al., 1998)) is activated in this subset of follicle cells and they adopt a dorsal fate. Thus, the DV axis is established perpendicular to the primary AP axis and by the same signalling mechanism (Gonzales-Reyes et al., 1995).

The establishment of DV polarity of the egg is manifest in the overall shape of the egg, the dorsal surface is flatter than the ventral surface and the dorsal appendages, the respiratory structures of the egg, are located dorso-laterally and anteriorly. The formation of these dorsal-anterior structures is dependent on an interaction between the dorsal signal, *grk*, and the anterior signal *decapentaplegic* (*dpp*) (Peri and Roth, 2000). The embryonic DV axis is determined by information built into the perivitelline space (Stein et al., 1991; Grosshans et al., 1994; DeLotto and DeLotto, 1998; Misra et al., 1998). The establishment of polarity in both the embryo and the eggshell relies on a common initial signal from the oocyte received by the anterior-dorsal follicle cells (Neuman-Silberberg and Schüpbach, 1993). This signal is responsible for activating different genetic pathways, which result in morphological differentiation in the egg and embryo.

The induction of a subset of follicle cells to adopt a dorsal fate is dependent on the correct localisation of the *grk* mRNA in the oocyte. However, we do not know in detail how the follicle cells respond once the *grk* signal is received to pattern the eggshell. Nor do we know exactly how this leads to the ventral follicle cells establishing the ventral signal in the perivitelline space, which is needed to establish the embryonic D/V axis. Towards this goal we have identified a number of genes expressed in specific subsets of follicle cells using the *GAL4/UAS* enhancer trap system (Brand and Perrimon, 1993; Deng et al., 1997). This paper describes the analysis of one of the genes isolated from this screen.

2. Results

2.1. Isolation of the loco *gene and determination of its expression pattern in oogenesis*

One of the enhancer trap lines, C139 exhibited *lacZ* staining in the anterior-dorsal follicle cells. This line was plasmid rescued and 2.3 kb of genomic DNA flanking the P-element was recovered. This genomic fragment was used to screen a *Drosophila* λ fix genomic library. Two λ clones were isolated (λ652 and λ653), which contained DNA 5′ and 3′ of the P-element insert. These λ clones were used to screen a *Drosophila* ovarian cDNA library. Two cDNAs were isolated, 96a and 118a. Restriction enzyme digests of the two λ clones and Southern blotting, using the rescued fragment and the two cDNAs as probes, showed that the λ clones spanned a region of 20 kb. The positions of the P-element, the rescued fragment and the two cDNAs are shown in Fig. 1A.

2.2. The cDNAs isolated represent transcripts of the RGS gene loco*, including a novel transcript*

The two cDNAs isolated from the library screen were fully sequenced. They represented different transcripts of the same gene and had sequence similarity to a newly identified family of RGS genes involved in G-protein regulation. Independently Granderath et al. (1999) identified transcripts for the same gene, *loco*. cDNA 118a proved to be identical

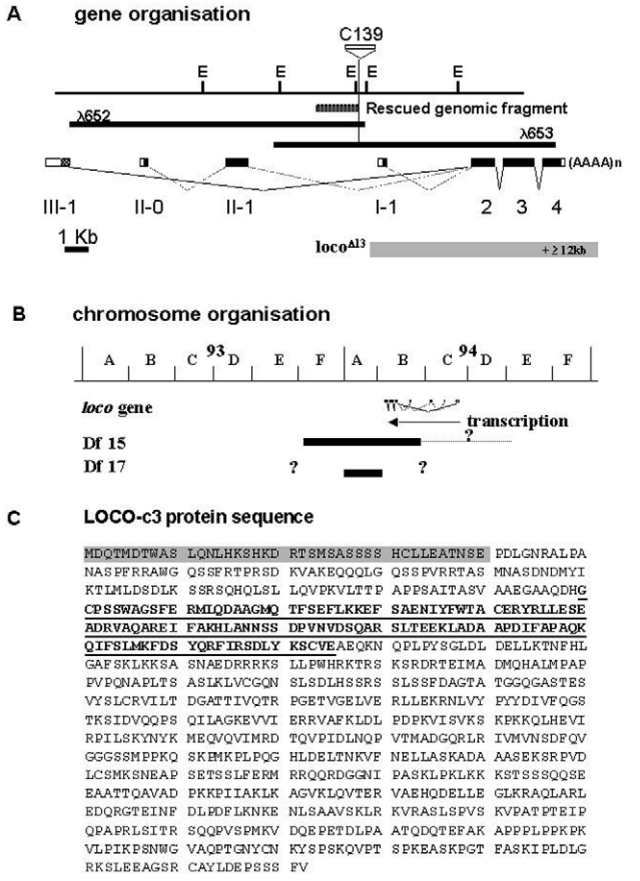

Fig. 1. (A) The genomic organisation of *loco*, showing the relationship between the genomic rescued fragment from fly line C139 and the 2 λ clones λ652 and λ653. Below this are the transcripts, with the exons as bars and the introns as a line and the coding regions shaded in black. The dotted line between the exons represents the linkages between the two transcripts of *loco* previously identified. *loco-c1* consists of exon I-1–4, *loco-c2* consists of, II-0, II-1–4. In keeping with the established numbering system, the transcript we have identified is *loco-c3*, and has its first exon about 2.5 kb upstream of exon II-0 and is spliced directly to exon 2, with a 15 kb intron separating these exons. The 5′ exon of *loco-c3* has been numbered III-1 and is 775 bp long, the majority of this is 5′UTR. (B) Genomic region 93–94. The location of the *loco* gene at 94 BC and the deficiencies *Df(3R)15CE1* and at *Df(3R)17D1* are indicated. The DNA deleted is a solid line and the dotted lines represent the possible maximum extent of their deficiencies. (C) Protein sequence of Loco-c3, where the shaded area is the translated region of exon III-1, unique to transcript three. The highlighted and underlined area is the conserved sequence of *loco* and represents its functional domain.

to the transcript *loco-c2* which they described. The other transcript cDNA 96a contains the same core region as the two transcripts described in Granderath et al. (1999), including exons 2–4, but contains a completely different 5′ exon of 800 bp (Fig. 1C); this transcript was named *loco-c3*. Reverse transcriptase polymerase chain reaction (RT-PCR) performed on ovarian tissue confirmed that the cDNAs represent genuine ovarian transcripts. RT-PCR also confirmed the presence of *loco-c1* in oogenesis. Mapping of the exon/intron boundaries was undertaken using a mixture of sequence alignment of cDNAs to genomic sequence in the NCBI database and PCR, using the two λ clones as templates, along with the corresponding genomic DNA. The full region spanning the gene has been added to the database from the Genome Project (AC 017222) and confirms our data. The new exon we identified in *loco-c3* maps to a region 2.5 kb upstream of exon II-0; this means that the intron between exon III-1 and exon II-0 of *loco-c3* is just over 15 kb (Fig. 1). The first exon, III-1, of *loco-c3* is 800 bp, 120 bp of which are coding. The start codon is 120 nucleotides from the 3′ end of the exon, indicating that this exon is largely untranslated.

2.3. The two transcripts present in ovaries have different expression patterns

Having established that there are at least two *loco* transcripts present in oogenesis, in situ hybridisation to wild type ovaries was carried out with transcript specific RNA probes to investigate the spatial and temporal expression of the different transcripts. To detect *loco-c2*, we used a probe consisting of exon II-1. Expression is first seen in the germarium at stage 2, in the prefollicular mesoderm surrounding the cystocytes (Fig. 2A). The prefollicular mesoderm goes on to form the follicular epithelium (King, 1970; Fig. 2A). Expression is then observed in the follicle cells at stage 8 (Fig. 2B). From stage 10 onwards *loco-c2* transcripts are present in the anterior-dorsal follicle cells and persist there until the follicle cells degenerate (Fig. 2C–E). There appears to be a higher level of expression in the dorsal midline follicle cells and at the margin between the oocyte and nurse cells, than there is laterally (Fig. 2D, F). Note that there is distinct difference in the level of transcript in adjacent cells rather than there being a gradient across this area.

In situ hybridisation to *loco-c3* RNA, using exon III-1 as a probe, demonstrates that the expression pattern is initially similar to *loco-c2*, as we detect transcripts in the germarium (Fig. 2E). The transcript is then undetectable until stage 6 when *loco-c3* transcripts are observed in the nurse cells. Levels increase dramatically at stage 10 (Fig. 2F) and transcripts persist until the nurse cells degenerate. Clearly the two *loco* transcripts detected in oogenesis are very differently regulated.

2.4. Expression of loco in follicle cells is downstream of the gurken-torpedo signalling pathway

Since *loco-c2* transcripts were observed in the anterior-dorsal follicle cells, we investigated if their expression was regulated by the EGFR signalling pathway. In situ hybridisations to *loco* RNA (probe for all exons used) in ovaries from a grk^{HK} mutant were undertaken and no *loco* expression was observed in follicle cells, though the nurse cell expression remained unchanged (Fig. 3A). Similar results were obtained using *torpedo* mutant ovaries (Fig. 3B). In an *fs(1)k10* mutant background (Haenlin et al., 1987), where *gurken* mRNA is not localised, we observed expression of *loco* extending further ventrally (Fig. 3C), and in a transgenic fly line that overexpresses *gurken* because it carries four copies of the gene (Neuman-Silberberg and Schüpbach, 1994), *loco-c2* expression is seen in all the anterior follicle cells (Fig. 3D). These results indicate that expression of the *loco-c2* transcript is downstream of the *gurken-torpedo* signalling pathway in follicle cells, and therefore the expression pattern is governed by the *gurken* signal from the oocyte to the overlying follicle cells.

2.5. Is the expression of loco regulated by defaultpointed in oogenesis?

As well as being isolated in our screen for genes involved in DV axis determination in the oocyte, *loco* was identified in a screen for genes downstream of *pointed* in specific subsets of cells in the central nervous system (CNS) (Granderath et al., 1999). *pointed* 1 and 2 are expressed in the anterior-dorsal follicle cells in oogenesis (Morimoto et al., 1996). The expression of *pointed* in oogenesis is dynamic, expression first being observed in the germarium, then later at stage 8, downstream of *torpedo*, in the posterior follicle cells and again at stage 10, also downstream of *torpedo*, in anterior-dorsal follicle cells (Morimoto et al., 1996). As *pointed* is observed in anterior-dorsal follicle cells in a similar pattern to *loco* at stage 10, it is possible that *loco* is downstream of *pointed* at this particular stage of oogenesis. We therefore analysed the relationship between *loco* and *pointed* in these cells. Using a *pointed 1/UAS* sense fly line, we drove expression of *pointed* in all the follicle cells using a T155 GAL4 driver. Fig. 4A shows an in situ hybridisation to RNA in the *GAL4/UAS-pointed* ovaries, using a *pointed* probe, and clearly shows high levels of *pointed* expression in all the follicle cells that cover the oocyte at stage 10. We then followed the expression of *loco-c2* in egg chambers where *pointed* was being ectopically expressed. We saw a normal spatial distribution in anterior-dorsal follicle cells at stage 10, though levels of expression were somewhat reduced (Fig. 4B). This reduction in expression is not uniform over the anterior-dorsal region, with the anterior-most follicle cells maintaining their normal level of expression. At later stages the expression pattern is slightly different to wild type as there is a

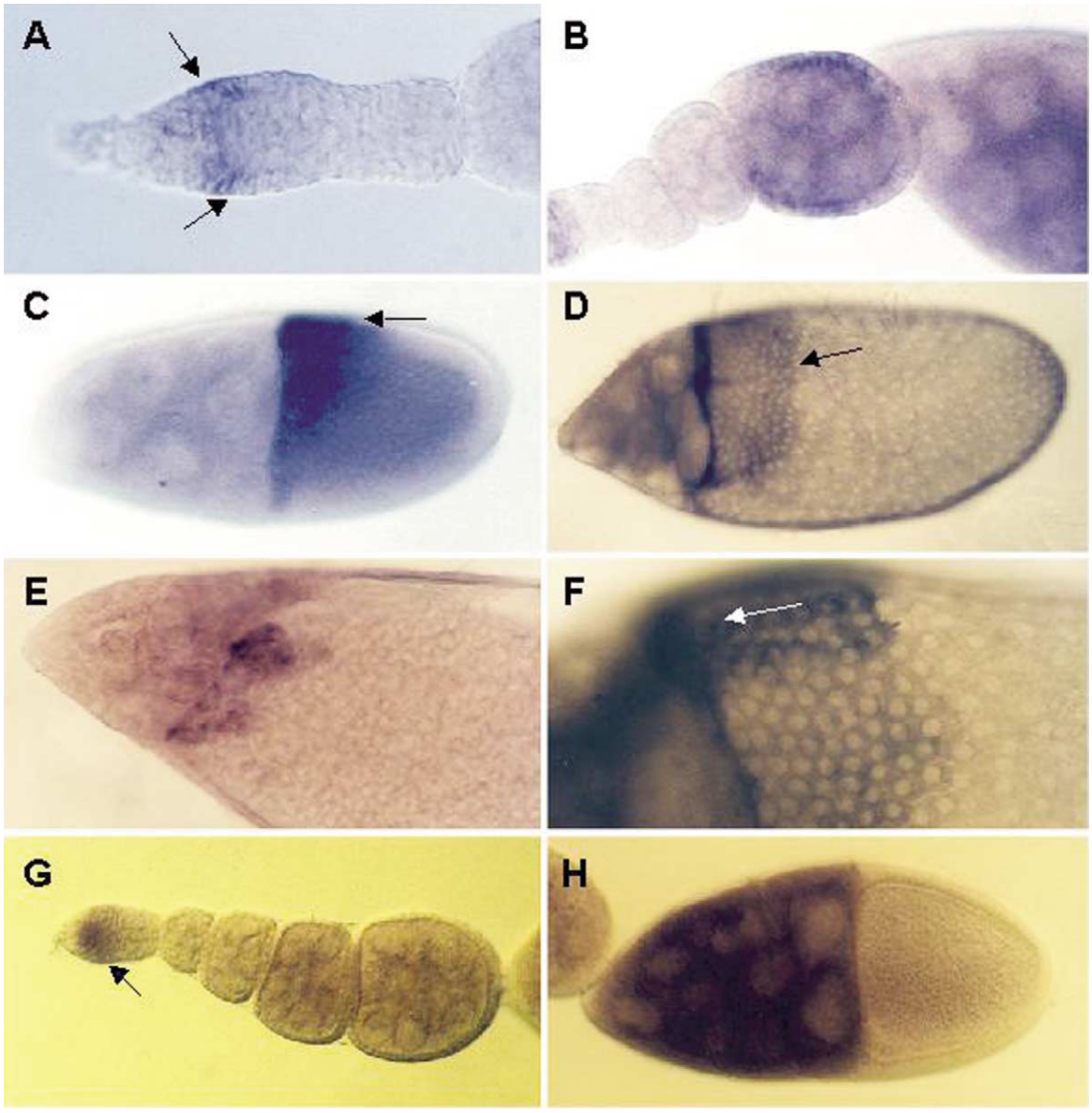

Fig. 2. (A–H) In situ hybridisation to ovaries using DIG labelled RNA probes made from the *loco* cDNA. The dorsal midline is in each case marked by an arrow. The expression of two *loco* mRNA transcripts found in oogenesis, *loco-c2* and *loco-c3* have been investigated by in situ hybridisation with RNA probes. (A–F) show the results with an exon II-1 probe, specific to transcript *loco-c2*. *loco-c2* mRNA is first observed in the germarium at stage 2, in the prefollicular mesoderm surrounding the cystocytes (A), it is then present in stage 8 egg chambers at a low level, in most of the follicle cells (B). *loco* is then observed in the anterior-dorsal follicle cells from stage 10 onwards (C) (also see Fig. 1A–D). (F) The dorsal-anterior follicle cell region of (A) magnified. It can clearly be seen in this that there is more *loco* expression in the dorsal midline cells and the anterior-most follicle cells. At stages 13–14 this expression is reduced to a small group of the anterior-most follicle cells (D). The expression pattern observed in anterior-dorsal follicle cells suggests that the cDNA could be downstream of the *gurken-torpedo* signalling pathway. To detect the *loco-c3* transcript, a probe specific to exon III-1 was used. *loco-c3* mRNA is observed in the germarium (G) after which it is not observed until stage 10, where it is expressed in all the nurse cells and (H) persists in the nurse cell until they degenerate at stage 14.

patch of cells expressing *loco* in the dorsal position, which has not migrated as far anteriorly as would be expected at this stage. This is due to overexpression of *pointed* in oogenesis resulting in failure to make dorsal appendages. This suggests that cells normally expressing *loco* do not migrate as far as in wild type egg chambers (Fig. 4C). This experiment shows that there is not a simple relationship between *loco* and *pointed*. Granderath et al. (2000) have shown clearly that *pointed* and *gcm* synergistically control *loco* in glial cell formation, so it is likely that there are other genes involved in regulating *loco* expression in the ovary. Ectopic *pointed* expression does disrupt the normal *loco-c2* expression pattern at stage 10, with its level of expression dropping in the anterior-dorsal follicle cells, except in the anterior-most follicle cells. This indicates that *loco-c2* is downstream of *pointed*, although not directly.

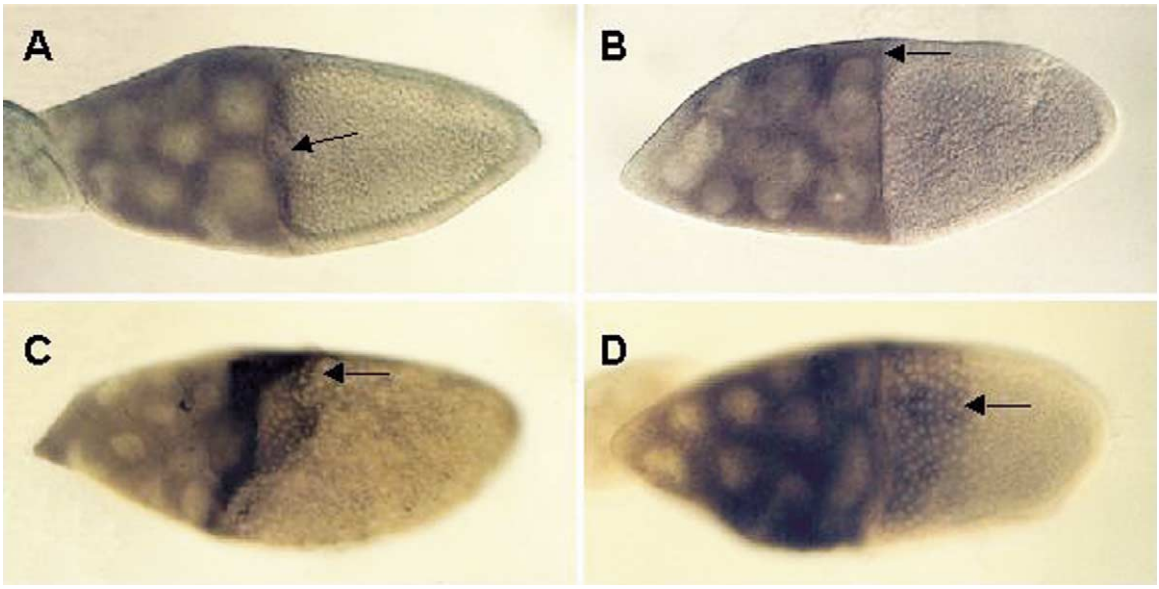

Fig. 3. (A–D) Egg chambers from different mutants. (A) A stage 10 egg chamber from a *gurken* (grk^{HK}) mutant line. Without *gurken*, Torpedo cannot be activated in the follicle cells. The dorsal-anterior expression of cDNA 96a is lost. (B) A stage 10 egg chamber from *torpedo* (top^{QY1}) mutant fly line. Again no expression of the cDNA is observed in the follicle cells. (C) A stage 10 egg chamber from an *fs(1)K10* mutant fly line. *fs(1)K10* is essential for the correct localisation of *gurken* mRNA to the oocyte nucleus, we observe expression of *loco* mRNA in the follicle cells, although it is not confined to the anterior-dorsal follicle cells, but its expression extends ventrally correlating with distribution of *gurken* mRNA extending ventrally. (D) A stage 10 egg chamber from a fly line which overexpressed *gurken*, having four copies of the *grk* gene (Neuman-Silberberg and Schüpbach, 1994). *loco* mRNA is observed in more cells than in wild type. All the evidence indicates that *loco* lies downstream of *grk* and *top* and is positively regulated.

2.6. Generation and analysis of mutants

The P-element used to isolate the gene was mapped initially by Southern hybridisation of the rescued fragment to restricted λ clones. Sequencing of the rescued fragment places it 280 bp 5′ of exon I-1 (Fig. 1). Interestingly, this is in a similar location to the P-element insertion obtained by Granderath et al. (1999).

P-element mutagenesis was carried out on fly line C139 selecting for potential aberrant excisions (white-eyed lines). One hundred and seven white-eyed lines were generated, of which nine were homozygous lethal. All lines were screened by Southern analysis, using genomic fragments as probes surrounding the P-element. Three lines showed band shifts relating to aberrations in the vicinity of the original P-element. S94ME371, a largely lethal line, which generates occasional homozygous females has a partial deletion and duplication of the P-element resulting in an 8 bp duplication of genomic DNA 5′ of the P-element. Homozygous females from line S94ME371 lay eggs that exhibit a range of phenotypes that can be divided into two groups. The short egg phenotypes are a result of disrupted cytoplasmic dumping from the nurse cells into the oocyte and result in eggs ranging from slightly shorter than wild type to a quarter of the expected length (Fig. 5D). The other group of phenotypes include eggs where the D/V polarity is affected, resulting in eggs which have fused dorsal appendages to varying degrees. This ranges from eggs with dorsal appendages slightly closer together, eggs with appendages fused at the base, eggs with appendages fused along the

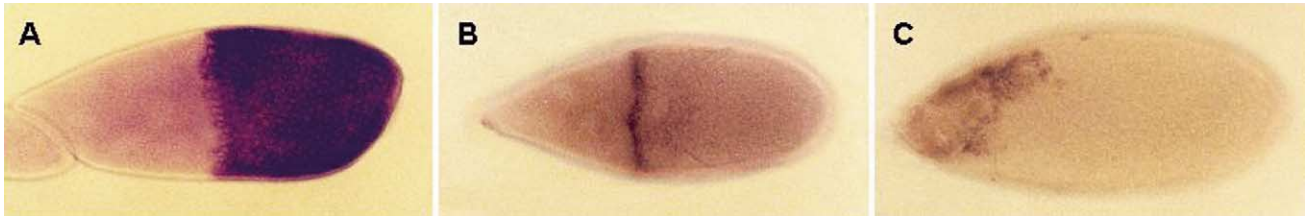

Fig. 4. The relationship between *loco* and *pointed* during oogenesis was investigated. (A) A stage 10 egg chamber from a UAS *pnt* sense line, in which *pnt* is being driven by C710 a *GAL4* driver for all the follicle cells. In situ hybridisation with a *pnt* probe shows that *pnt* is expressed in all of the follicle cells. (B, C) *loco-c2* expression in egg chambers with ectopic *pnt* expression. At stage 10 the expression pattern of *loco-c2* is reduced in all anterior-dorsal follicle cells except for those at the margin between the anterior of the oocyte and the nurse cells (B; this is a dorsal view). (C) *loco-c2* expression at stage 13, this is markedly different from wild type (compare with Fig. 3D).

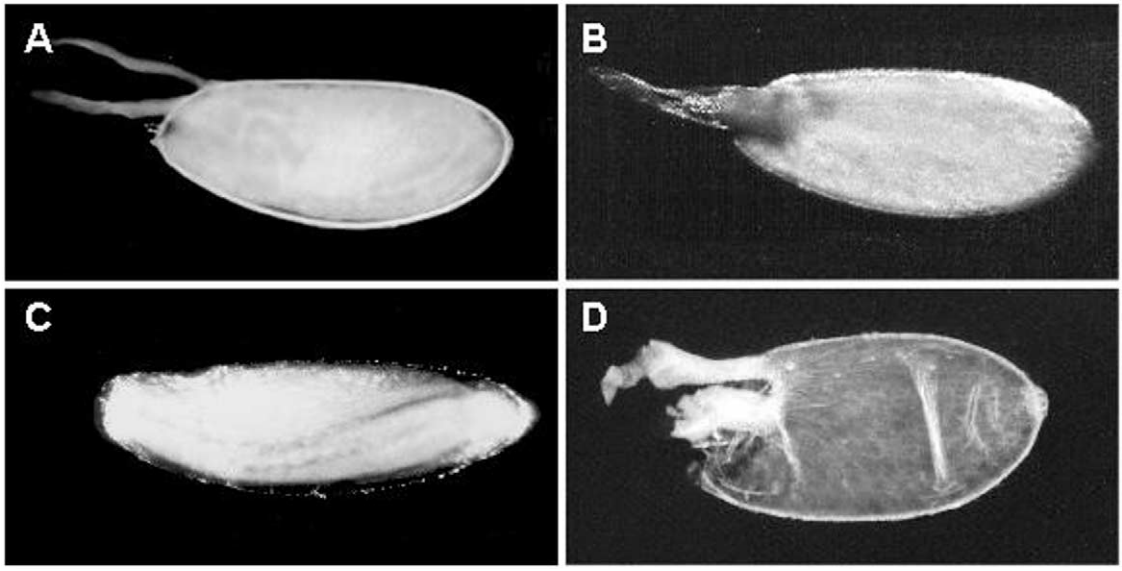

Fig. 5. The phenotypes observed in the mutant lines. (A) A lateral view of a wild type egg chamber. (B–D) are the various phenotypes observed in the mutant line *loco*^{371}. (B) A fused appendage phenotype, (C) a ventralised egg with no dorsal appendages and (D) a short egg, the result of cytoplasmic dumping not occurring properly.

entire length (one appendage), to eggs with no appendages (Fig. 5B, C). This can be interpreted as a reduction or loss of dorsal identity. The embryos that were lethal failed to develop cuticular structures and were generally degenerated. This is dealt with in the subsequent section.

loco$^{\Delta 13}$, a lethal *loco* mutant, and deficiencies *Df(3R)15CE1* and *Df(3R)17D1*, deleting the cytological regions 93E/F-94C/D and 93E/F-94B/C, respectively, were used in complementation analysis with excision line S94ME371, now called *loco*^{371}. *loco*^{371} complements *Df(3R)15CE1*, *Df(3R)17D1* and *loco*$^{\Delta 13}$. This was an unexpected result, as the molecular data for *loco*^{371} demonstrate that there is a disruption of the *loco* gene and *loco* has been mapped to 94B/C where the deficiencies lie. The organisation of *loco* and the positions of the *Df* lines are shown in Fig. 1B. *loco*$^{\Delta 13}$ is a deficiency line with a deletion (12 kb) extending from exon I-1 3′ of *loco*. Since *loco* is in the orientation $3' \rightarrow 5'$ with respect to the chromosome numbering system the defect in mutation in *loco*^{371} clearly maps outside this deletion and could therefore complement by virtue of producing some transcripts. The position of the 8 bp insertion is likely to disrupt the regulation of some *loco* transcripts rather than causing the transcripts to be absent. Similarly, the two *Df* lines include the *loco* complementation group and clearly delete *loco* core exons, but they complement our mutations in the 5′ region of the *loco* gene. We propose that *loco*^{371} disrupts expression of *loco* in such a way that two copies of a regulatory mutation, as in the homozygotes, is worse developmentally than having one copy, as seen when crossed to *loco* deficiencies. This is entirely possible if *loco*^{371} is causing ectopic expression or abnormal expression of some isoforms.

2.7. Investigating the role of loco *in oogenesis using antisense RNA*

Antisense *loco pCaSpeR-hs* and *pUAST* constructs were made and used to generate heatshock and UAS fly lines. As *loco-c2* is expressed in the anterior-dorsal follicle cells, disruption of *loco* here may result in either DV defects in egg patterning, DV defects in embryo patterning or both. The eggs laid by the *GAL4/UAS* antisense flies were observed and defects in the eggs described. Two lines exhibited a weak ventralised phenotype, 96UAS-ve6a laying some eggs with fused appendages and 118aUAS-ve laying some eggs with fused appendages and ventalised eggs. The weak phenotype observed probably results from low levels of antisense expression not removing all *loco* function. The lack of effect in several lines can be attributed to positional effects (Deng et al. 1999).

Analysis was carried out on all the *pCaSpeR-hs* lines. After heatshock, eggs were collected and observed to see if there were any abnormalities in the eggs laid. Of the 17 lines observed two were studied in detail as they exhibited the strongest phenotypes. Under the same heatshock regime no such egg abnormalities were observed with the wild type control (OrR). 96hs-ve5A produced eggs which either had fused appendages (14%) or appendages were completely absent and the egg was ventralised (4%). The heatshock data produced corresponded with *GAL4/UAS* data given above in that abnormal phenotypes included a range of dorsal defects, from eggs with slightly fused appendages to eggs which are completely ventralised (Fig. 6D, F, H).

The other line analysed in detail was 96hs-ve2A where 9% of eggs laid were shorter than wild type. The short egg phenotype observed in these lines varied from slightly

shorter than normal eggs to eggs that were approximately a quarter the length of wild type eggs (Fig. 6E). A short egg phenotype is often associated with failure of the nurse cells to dump their contents at the end of oogenesis (Cooley and Theurkauf, 1994). Since we observe expression of *loco* in the nurse cells these phenotypes could result from disruption of *loco* function in nurse cells.

The two different phenotypes resulting from two transgenic lines 5A and 2A required further investigation as both fly lines contained the same hs-antisense-*loco* construct. This could indicate that the activation of the heatshock construct results in the misexpression of another transcript or creates a fusion product in one of the heatshock lines. The organisation of the vector makes the latter unlikely and the misexpression of a new transcript is also unlikely as two antisense lines with insertions in different chromosomal positions give the same short egg phenotype. Another possibility is that as the different heatshock lines are inserted into different chromosomal locations the heatshock induces antisense *loco* in different cells at different developmental stages, and this could account for the observed differences. To investigate this possibility further, in situ hybridisation to the antisense *loco* RNA in the heatshock lines, following the heatshock regime used to generate this phenotype was undertaken. The heatshock lines did not express antisense-*loco* ubiquitously, and the two lines generating the different egg phenotypes had different antisense-*loco* expression patterns. 2A had germ-line specific expression in the ovaries (Fig. 6A), while 5A had expression in the follicle cells overlying the oocyte at stage 10 (Fig. 6B). This confirms that there are positional effects, due to the insertion site of the heatshock construct. This differential expression proves very useful as the individual heatshock lines are effectively interfering with the somatic and germ-line transcripts of *loco* separately. Line 5A drives follicle cell expression and generates the same phenotype as the *GAL4/UAS* cross that also drives antisense *loco* in the follicle cells. This disrupts *loco-c2* in the anterior-dorsal follicle cells and results in eggs with dorsal-anterior defects. Disruption of *loco-c3* in the nurse cells as seen in line 2A, however, results in a dumpless phenotype. The results observed are therefore consistent with antisense-*loco* interfering with *loco* function in the two sites of antisense expression.

As heatshock induces antisense RNA for a short period of time in either the germ-line or follicle cells in 2A or 5A, respectively, we used this system to investigate at which stage in oogenesis the two functions of *loco* are required. The time elapsed between heatshock and eggs with abnormal phenotypes being laid was determined. For line 2A short eggs were observed 15–20 h after heatshock at a frequency of 9% (Fig. 6I). This correlates with the oocytes that form these abnormal eggs receiving the heatshock at stage 10 of oogenesis. This would disrupt *loco* when it is present in both follicle cells and nurse cells. Using line 5A the fused appendage phenotype is first observed in deposited eggs approximately 19 h after heatshock, followed by the ventralised phenotype at 23 h (Fig. 6J). This suggests that egg chambers that receive the heatshock at or before stage 10 go on to be fully ventralised, while the egg chambers that receive the heatshock after stage 10 only have fused appendages. Control experiments subjecting wild type flies to the same heatshock regime did not generate these phenotypes. All these observations confirm that the phenotypes observed are a product of antisense disrupting native transcript as opposed to an artefact of the heatshock regime. We have also generated antisense *fringe* transgenic heatshock lines that exhibit different phenotypes after heatshock to those we observe in the *loco* antisense heatshock transgenic lines (Zhao et al., 2000). These *fringe* lines also have different spatial antisense expression consistent with our observations in the *loco* heatshock lines. These observations show the specificity of the antisense technology.

2.8. *Is* loco *involved in DV patterning of the embryo as well as the egg?*

The egg phenotypes we observe with both the mutant lines and the heatshock line showed loss of dorsal regions of the eggshell and range from fused appendages to eggs that completely lack dorsal appendages. In the eggs that entirely lacked dorsal appendages there was a distinct difference in the overall shape of the egg, which appeared symmetrical, with the operculum missing. The majority of the eggs with fused appendages hatched into healthy larvae but the ventralised eggs failed to hatch. The embryos inside these eggs arrested early in development, and had little morphological structure. This suggested that maternal *loco* is essential for embryonic development. To ascertain if the embryos that failed to develop were also ventralised we investigated the expression of *twist*. *twist* transcripts can be used as a marker for ventral embryonic cells, and is activated very early in embryogenesis, as soon as the epithelium is formed (Fig. 7B). It persists in the ventral cells until late in development. *twist* in situ hybridisation on 0–4 h, blastoderm stage OrR embryos shows a band of staining in the ventral cells (Fig. 7A). This band reaches a maximum width of 18–20 cells (Fig. 7B). Amongst the abnormal embryos laid by fly line $loco^{371}$, some have an increased width of *twist* expression with the band being in excess of 24 cells (Fig. 7C,D). Blastoderm embryos were also observed in which the *twist* expression was disrupted; in some *twist* expression was patchy and expanded at the anterior and posterior poles (Fig. 7E), in some expression was completely absent in the posterior half of the embryo (Fig. 7F). The results showed that normal *twist* expression is disrupted in embryos laid by mutant *loco* mothers, confirming that *loco* is not only involved in the establishment of the axis of the egg, but also required to establish the axis of the embryo.

2.9. *Development of embryos laid by* loco *mothers*

Since the embryos that die have little morphology at the

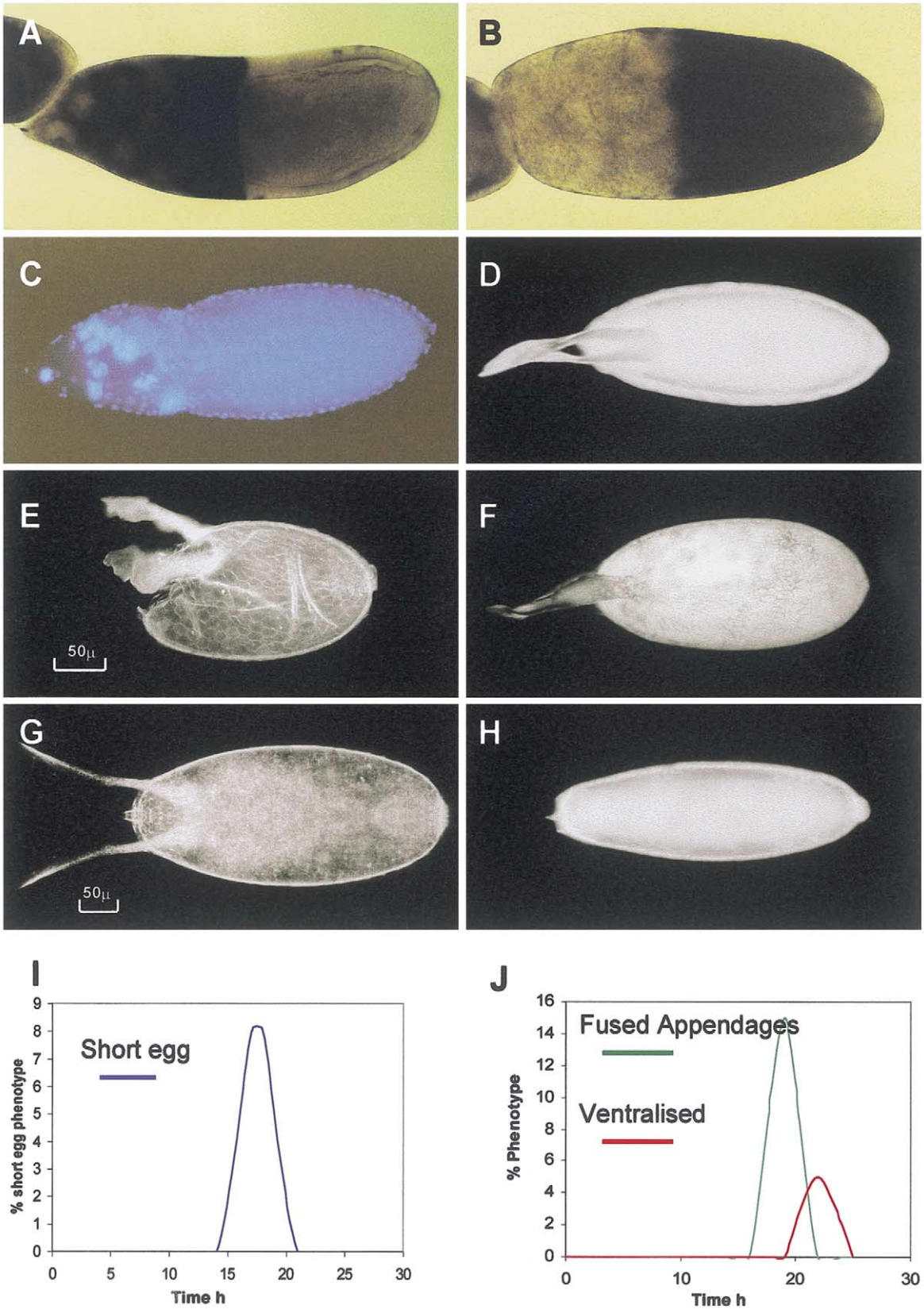
A
B
C
D
E
F
50μ
G
50μ
H
I
Short egg
% short egg phenotype
Time h
J
Fused Appendages
Ventralised
% Phenotype
Time h

Fig. 6.

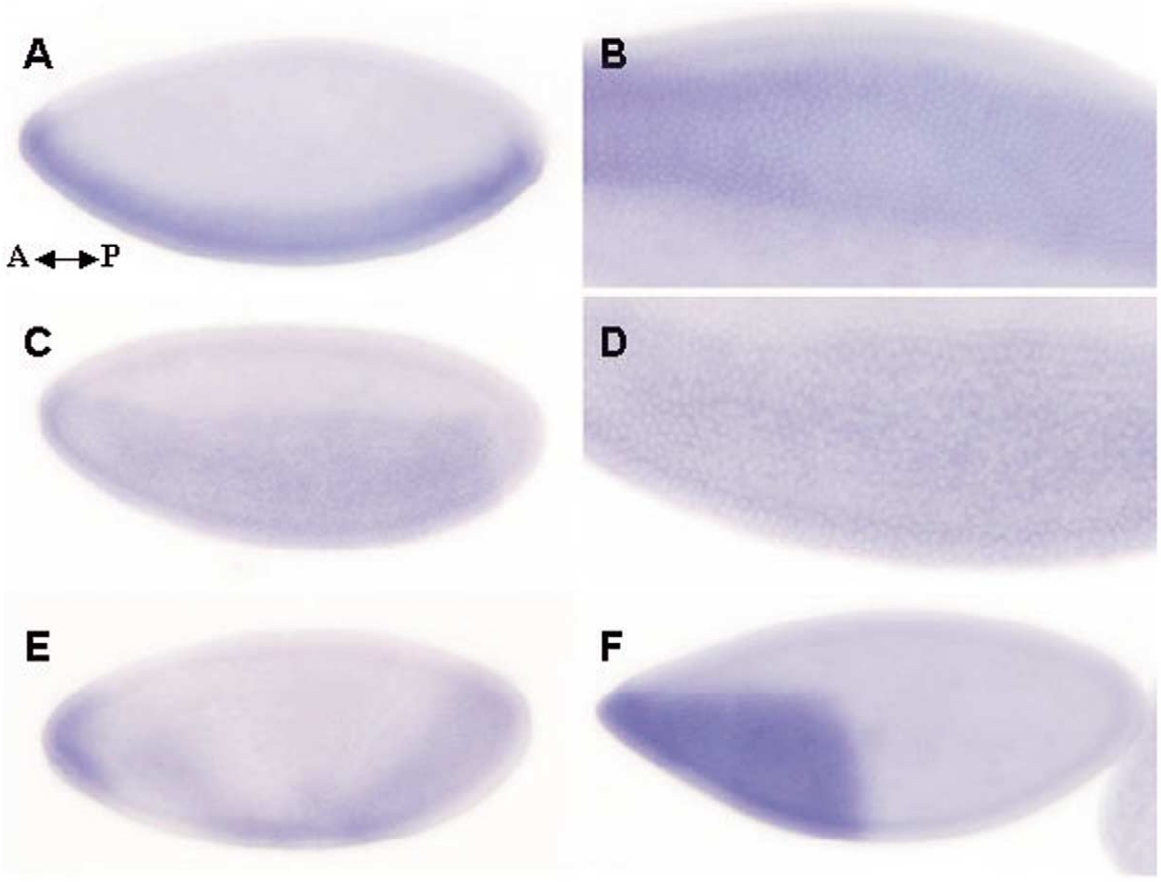

Fig. 7. (A) A wild type embryo, which had been hybridised to a *twist* RNA probe. *twist* expression can clearly be seen along the ventral surface of the embryo. (B) A ventral view of a similar embryo, showing width of the staining (18–20 cells). (C–F) are embryos laid by homozygous $loco^{371}$ mutant mothers. (C) A lateral view of an embryo from $loco^{371}$, this shows a broader band of expression than observed in any of the wild type embryos. (D) Part of (C) at higher magnification, showing expression spanning at least 24 cells. In (E) the expression of *twist* is more patchy and extends further dorsally at the poles. In (F) *twist* expression is lost in much of the blastoderm and is limited to the anterior.

time they should hatch we collected eggs from homozygous *loco* mothers in 4 h batches and observed their development. Nuclear multiplication occurred and the blastoderm began to cellularise. In some instances, cellularisation was not even across the entire surface and some eggs produced abnormal blastoderms (Fig. 8A2), a common defect was a failure of correct invagination of the gut. Invagination began too far towards the posterior and large 'holes' appeared in the central yolk region (Fig. 8B3–4). These embryos gradually became more abnormal as development proceeded and eventually degenerated without secreting cuticle.

Other abnormalities were observed, where midgut inva-

Fig. 6. (A) A stage 10 egg chamber from fly line 96hs-ve2a (an antisense *loco* heatshock fly line) after it had been exposed to a heatshock (HS) regime. RNA in situ hybridisation with a probe against the antisense strand of *loco* shows that this line only drives antisense *loco* expression in the germ-line. In (B) a similar experiment was carried out using 96hs-ve5a (another antisense *loco* line carrying the same construct as in (A)). In this line HS only drives antisense *loco* expression in the follicle cells. As both lines contain the same construct, the difference in expression patterns must be due to position effects. (C) The ovarian phenotype observed in line 96hs-ve2a after the HS regime. This is a dumpless phenotype, and results in small eggs being laid (E). Note (G) shows a wild type egg and is not of the same scale as (E). (I) The numbers of short eggs laid by line 96hs-ve2a following the HS regime. It illustrates that the phenotype was observed 15–20 h after HS. Phenotypes (D), (F) and (H) are laid by in 96hs-ve5a females following HS treatment. (D) is the weakest phenotype observed with the dorsal appendages fused at the base. In (F) there is a single fused appendage in the middle, both (D) and (F) are representative of a reduced dorsal area in the eggshell. (H) shows the most severe phenotype, which is a complete loss of dorsal appendage. There is also a change in the shape of the egg, which is longer and thinner. (J) The numbers of eggs laid with fused appendages and with ventralised eggs following HS of line 96hs-ve5a. It illustrates that the fused egg phenotype was first observed 19 h after HS followed by the ventralised phenotype 23 h after HS. The fused appendage phenotype was more abundant than the ventralised phenotype, presumably reflecting a smaller developmental window when the HS can affect DV axis determination compared to affecting appendage determination and differentiation.

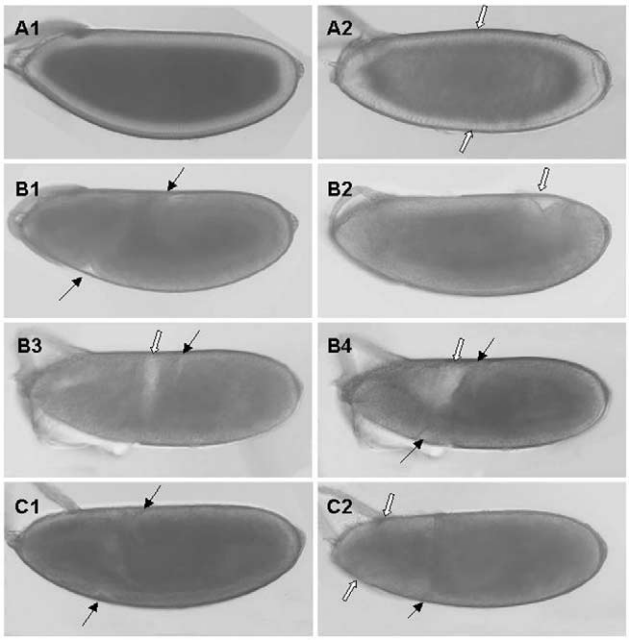

Fig. 8. Mutant phenotypes observed. All mutant embryos illustrated are the progeny of virgin *loco*^{371} females crossed to OrR males, therefore any embryonic defects observed can be attributed to disruption of maternal *loco*. The black arrows mark the gut invaginations and the white arrows abnormal regions. (A1) shows a wild type mid-stage 5 embryo after cellularisation has occurred. In the mutant (A2) cellularisation has occurred, but the cytoplasm has not cleared properly. Such embryos if left to develop become highly disorganised and fail to show any signs of germ band extension or gastrulation. (B1) A wild type stage 8 embryo during germ band extension, (B2–4) show embryos laid by *loco*^{371} mothers at a similar stage. In (B2) midgut invagination is not occurring properly. (B3, B4) The same embryo at 4 and 6 h into development, respectively. Gut invagination is not occurring properly, failing to move as far anteriorly as it should, resulting in a hole in the middle of the embryos. These embryos do not progress beyond this stage. (C1) A normal late-stage gastrula and C2 an embryo with an abnormal dorsal head region. The range of patterning defects observed correspond to *twist* embryo in situ hybridisations illustrating that when maternal *loco* is disrupted it results in disruption of events that depend upon normal DV axis formation.

gination did not occur properly (Fig. 8B2). Embryos were also observed with abnormal head regions that failed to show any signs of head invagination (Fig. 8C2).

3. Discussion

The data in this paper demonstrate that the RGS gene *loco* is expressed in *Drosophila* oogenesis. There are at least two different transcripts present, which are expressed in different subsets of cells at different stages of oogenesis and are involved in different developmental processes. Analysis of UAS and heatshock induced antisense expression and *loco* mutants, along with molecular studies, enabled us to match transcripts with specific developmental mechanisms.

The egg phenotypes observed laid by *loco*^{371} homozygous females suggest a role for *loco* in DV axis formation of the egg. This data was corroborated by heatshock induced antisense-*loco* experiments. However, the mutant analysis was not straightforward. Although our molecular studies clearly show that we have generated mutants in the *loco* gene and there are no other P-elements in the stocks, the complementation analysis did not show a more severe phenotype when our newly generated mutants were crossed to the existing deficiencies in the region. We know that *loco* is a large gene with several differently spliced forms, which would suggest a complex mechanism of gene regulation. Preliminary analysis with antibodies has also shown there are different isoforms of the protein present at distinct developmental stages suggesting different roles for different protein isoforms. As a negative regulator of G-protein levels, the types of protein expressed would be critical in maintaining equilibrium in signalling systems. All these factors could contribute to observed behaviour of *loco*^{371} which has a small insertion and retains rearranged parts of the P-element in the *loco* gene. This would be likely to interfere with expression of some transcripts but not others. This misregulation would lead to unusual complementation analysis, as having two copies of a gene which is expressed in the wrong cells or at the wrong time, or as the wrong isoform is likely to be more developmentally disruptive than a single copy. This would explain why homozygotes are mostly lethal and hemizygotes are not.

The heatshock antisense results were crucial for interpreting how the different mutant phenotypes were generated and which transcript and cell type they depended on. The different heatshock lines generate transcripts in different cell types. This allowed us to disrupt *loco* separately in the germ-line and follicle cells. *loco-c3* is expressed in the nurse cells and, when disrupted, results in a dumpless phenotype resulting in smaller than normal eggs being laid. When *loco-c2* is disrupted in the anterior-dorsal follicle cells, this results in a range of dorsal defects in the eggs. The anterior-dorsal egg defects vary from dorsal appendages, which are fused at the base, to appendages fused along the whole length, to completely absent appendages.

Our results suggest a role for G-protein signalling in DV pattern formation in oogenesis. Granderath et al. (1999) have carried out a two hybrid screen with *loco* and illustrated that Giα interacts with *loco*. This is direct evidence for a conserved role between *loco* and mammalian RGS genes.

In recent years, the study of the EGFR signalling pathway has highlighted how complex the DV patterning of the eggshell is. In oogenesis the proper formation of dorsal structures of the egg rely on this tight regulation. Rhomboid (Ruohola-Baker et al., 1993) is known to upregulate the initial activation of the EGFR signal by Gurken. Rhomboid is thought to act by cleaving Spitz, releasing activated Spitz (Schweitzer et al., 1995; Golembo et al., 1996; Wasserman and Freeman, 1998) which binds to Torpedo (EGFR) upregulating it. Another ligand of EGFR, Vein, is also thought to be involved in this process (Wasserman and Freeman, 1998). In this way, the initial paracrine signal from *gurken*

in the oocyte becomes an autocrine signal within the follicle cells.

The secreted protein Argos is produced following high levels of EGFR activation (Wasserman and Freeman, 1998; Zhao and Bownes, 1999). This secreted protein represses the EGFR signal, possibly by interacting with the external domain of EGFR preventing any further ligands from binding. Because Argos is a secreted protein it has been suggested that it diffuses from the region where it is made, resulting in gradient around the dorsal midline follicle cells. It is postulated Argos separates the single EGFR signal into two peaks resulting in two dorsal appendages (Wasserman and Freeman, 1998). The regulation of the EGFR signal does not stop here as Kekkon I, which is a transmembrane protein, is thought to associate with the external domain, thereby modulating the signal (Ghiglione et al., 1999). The product of another novel gene, *sprouty*, has been shown to negatively regulate the EGFR signal (Reich et al., 1999).

Why is there such an elaborate process to specify cell fates along this axis and how does *loco* fit into the existing pathway? *loco* is downstream of *torpedo* in the follicle cells and appears to be activated both at high and moderate levels of *torpedo* activation. The levels of expression are higher in the dorsal midline follicle cells and the follicle cells lying at the nurse cell/follicle cell junction. This suggests that *loco* expression is controlled by other genes expressed in the anterior-dorsal follicle cells as well as the Grk signal.

We know that the role of RGS proteins is to negatively regulate G-protein signalling. Granderath et al. (1999) have shown with a two hybrid screen that *loco* binds Giα. Wolfgang et al. (1991) showed Giα is present in the anterior-dorsal follicle cells. Thus it seems likely that G-protein signalling has a role in DV axis formation. In the absence of *loco* there is a loss of DV polarity in the egg and embryo. This phenotype is similar to the one induced by Gurken mutants and suggests that in the absence of *loco* repression EGFR signalling occurs. Since RGS genes negatively regulate G-protein signalling one can assume that in wild type ovaries G-protein signalling inhibits EGFR signalling but the presence of *loco* prevents this inhibition in anterior-dorsal follicle cells. This adds to the complexity of regulation of the EGFR pathway and suggests that the tight regulation or modulation of EGFR is critical for the correct sequence of morphological events to occur in the specification of follicle cell fates along the DV axis with time. This helps explain the range of *loco* phenotypes observed in the egg as the resulting phenotype will depend on the time and extent to which *loco* is affected. It also helps explain the weak phenotypes observed as there are several feedback loops occurring that presumably compensate to varying degrees for disruption of *loco* activity, trying to maintain correct EGFR signalling.

Granderath et al. (2000) showed that the relationship between *pointed* and *loco-c1* transcript is dependent on the gene *gcm*, and that *pointed* and *gcm* act synergistically to activate *loco-c1*. In the follicle cells it is *loco-c2* that is present. Our evidence suggests that *pointed* is involved in regulation of *loco-c2* in ovarian follicle cells, however, in a manner similar to the regulation of *loco-c1* in glial cells, other genes are probably involved in the regulation of *loco*.

Several different pathways are essential for the anterior-dorsal patterning of the egg. It has recently been illustrated that the operculum is patterned independently and its size can be affected by the genes *dpp* and *bunched* (Dobens et al., 2000). In addition an interaction is needed between *dpp* at the anterior and *gurken*, the dorsal signal, to form dorsal appendages (Peri and Roth, 2000). It superficially appears that *loco* affects operculum formation when observing the most severely ventralised eggs. However, the micropyle and a ridge of the operculum similar to that seen normally on the ventral anterior of the egg are visible on a *loco* ventralised egg. This indicates that the ability to form the operculum is still present, and *loco* is affecting DV axis determination.

The DV defects in *twist* expression observed in the embryos of eggs laid by fly line $loco^{371}$, help clarify the role of *loco* in oogenesis, showing that disruption of maternal *loco* in oogenesis results in the disruption of native *twist* expression, a marker for ventral cell fate, and clear DV defects are observed in the embryos that fail to hatch, as many fail to form normal guts. The range of patterning defects observed are related to the disruption of native *twist* expression. This suggests maternally expressed *loco* is required for normal embryonic development. We cannot distinguish if the failure of embryos to develop properly and the observed defects in *twist* expression in the early embryo result from the mutant follicle cells affecting the developing egg chamber and oocyte or the maternal contribution of *loco* from the nurse cells deposited in the egg being needed for zygotic development of the D/V axis. However, the *twist* expression observed only in the activation of the embryo does suggest that disruption of *loco* in anterior-dorsal follicle cells may affect embryonic patterning. Dorsal follicle cell identity is determined as the follicle cells migrate posteriorly over the egg chamber, coming into contact with the localised *gurken* signal over the oocyte nucleus. If the EGFR signalling was disrupted in follicle cells during the course of this migration, it would result in an embryo that only had abnormal D/V patterning along part of its AP axis.

3.1. The role of loco in nurse cell cytoplasmic dumping

The small egg phenotype observed in both the mutant lines and heatshock antisense lines indicates that *loco* is required for cytoplasmic dumping from the nurse cells to the oocyte. Several mutants have been identified that are involved in this process (Mahajan-Miklos and Cooley, 1994), and the phenotypes can be attributed to two defective processes. The first is the failure of the ring canals to grow, restricting the flow of cytoplasm from the nurse cells to the oocyte (Cooley, 1998). The second is a change in the actin

cytoskeleton, with the nurse cells failing to centralise the nurse cell nuclei (Guild et al., 1997). When this happens the nuclei block the ring canals preventing dumping. Which process requires *loco* during nurse cell dumping remains to be determined.

In this paper, we have identified two roles for the *loco* gene in oogenesis; cytoplasmic dumping and DV axis formation. Goα mRNA is present in oocyte and nurse cells and Gsα protein in follicle cells; Giα mRNA is present in nurse cells and the oocyte, with Giα protein present in stage 10 anterior-dorsal follicle cells and the oocyte (Wolfgang et al., 1991). The expression patterns of the different Gα subunits correlate with the expression patterns of the *loco* isoforms, further suggesting a role for G-protein signalling in nurse cell dumping and a link between G-protein signalling and the EGFR pathway in follicle cells.

In mammals there are several different Gα subunits, these Gα subunits are also present in *Drosophila*. In mammals the different Gα subunits are regulated by different RGS proteins. With the *Drosophila* Genome Project complete we have searched for additional *Drosophila* RGS genes, but none were found. As different *Drosophila* G-protein α subunits are likely to need regulation, it may be that the different transcripts of *loco* regulate different Gα subunits.

4. Experimental procedures

4.1. Stocks

Wild type flies were OrR. The grk^{HK} and grk^{WG} were provided by Nüsslein-Volhard (Schüpbach, 1987), *fs(1)K10* (Wieschaus et al., 1978) and top^{QY1} (one allele of EGFR, Schüpbach, 1987), pnt^{P1UAS}, pnt^{P2UAS} were obtained from the Bloomington Stock Center. *Df(3R)17D1*, *Df(3R)15CE1* and $loco^{\Delta 13}$ were obtained from Chistian Klambt. The GAL4 drivers T155 and CU1 and the fly line containing four copies of *gurken* transgene were obtained from T. Schupbach (Queenan et al., 1997; Neuman-Silberberg and Schüpbach, 1994). All flies were raised on standard cornmeal–yeast–agar medium at 25°C.

4.2. Library screening

Genomic rescued DNA, 2.3 kb, was obtained from fly line C139. This genomic rescued fragment was used to screen a *Drosophila* CantonS λFix genomic library (Stratagene). Two λ clones were obtained, λ652 and λ653. These were both used to screen an ovarian λZAPII cDNA library (a gift from Y.N. Jan, UCSF, San Francisco, CA, USA). Two cDNAs were obtained, cDNA 96a and 118a, which are 1.7 and 0.8 kb, respectively. Both cDNAs were incomplete and lacked their 3′ end. An expressed sequence tag (EST) database search was carried out with both cDNAs and one EST was identified, LD27000 (Accession No. AA941661). This was sequenced and contained the missing 3′ sequence. RT-PCR was carried out on ovarian tissue to establish the structure of the cDNAs.

4.3. Whole mount in situ hybridisation of ovaries

The whole mount in situ hybridisation was conducted as described by Zhao and Bownes (1999).

4.4. DNA sequencing

The dideoxy chain determination method was used initially in the form of a Sequenase 2.0 kit (US Biochemicals), followed by automated sequencing on Perkin-Elmer ABI 373A and 377A machines using dye labelled primers, then dye labelled terminator reactions. Sequenced fragments were assembled using GCG and GENE-JOCKEY software. Sequence analysis was done with GCG GAP, MAP, FASTA, TFASTA and PILEUP software.

4.5. RT-PCR

RT-PCR was carried out as described by Deng et al. (1999).

4.6. Constucts and P-element mediated germ-line transformation

The complete cDNA 96a was cloned into both pCaSpeR-*UAS* and pCaSpeR*HS* in the antisense orientation.

P-element mediated germ-line transformation was conducted as described by Deng et al. (1999).

4.7. Antisense analysis

Antisense constructs were made containing antisense core exons 2 and 3 in pCaSpeRhs, (Thummel and Pirrotta, 1991) under heatshock control and in pUAST under UAS control (Phelps and Brand, 1998). Several transgenic fly lines were generated.

The pUAST lines were crossed to the *GAL4* follicle cell drivers Cu1 and T155; both these fly lines exhibit *GAL4* expression in all follicle cells after stage 8 (Queenan et al., 1997). The GAL4/UAS progeny from the crosses were transferred into a cage on apple juice plates. The eggs on each plate were collected and observed.

For the CaSpeR HS lines, 50–100 flies were raised in each milk bottle at 25°C for 3 days and then transferred into a fresh bottle with yeast paste smeared on top of the cornmeal food and kept at 25°C for 32 h. The flies were then transferred into a cage on apple juice plates, the flies were heat-shocked at 38°C for 40 min. The flies were moved back to 25°C and the apple juice plates changed at 4 h intervals. The eggs on each plate were collected and observed.

4.8. Antibody production

A GST-*loco* gene-fusion was used to produce protein containing 175 aa of Loco core region (Pharmcia Biotech

gene fusion system). The antibody was raised in sheep at the Scottish Antibody Production Unit.

4.9. Western analysis

OrR embryos were collected over 24 h periods and were stored at −20°C. *loco* mutant embryos were collected by selecting unhatched embryos at 26 h.

Western blotting and ECL detection were carried out as described in Deng et al. (1999).

4.10. Observation of living embryos

Living mutant and wild type embryos were observed in KELF oil, which allows development to be followed with the chorion intact. This approach was taken as mutant embryos tended to degenerate by the time they should have hatched, making it difficult to determine the primary developmental defects. No defects were observed in wild type embryos.

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